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Chimeric coxsackie B3 virus genomes that express hybrid coxsackievirus–poliovirus 2B proteins: functional dissection of structural domains involved in RNA replication

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The 2B proteins of coxsackievirus and poliovirus (PV) share significant structural similarity and exhibit similar biochemical activities, namely inhibition of protein secretion and modification of membrane permeability. Both proteins contain two hydrophobic domains in the carboxy-terminal two-thirds of their sequence, of which one has the potential to form a cationic amphipathic α-helix. To gain more insight into the structural requirements of enterovirus protein 2B for its functioning in viral RNA replication, a chimeric cDNA approach was used. Chimeric coxsackie B3 virus (CBV3) genomes were constructed that expressed either the entire PV 2B protein or hybrid proteins in which specific segments of CBV3 2B were substituted by their corresponding PV counterparts. *In vitro* synthesis and processing of the chimeric polyproteins showed no abnormalities. CBV3 genomes carrying the entire PV 2B gene failed to replicate. A chimeric genome that expressed a hybrid 2B protein consisting of the amino-terminal one-third of PV and the remainder of CBV3 yielded viable viruses. In contrast, a 2B protein consisting of the amino-terminal one-third of CBV3 and the remainder of PV failed to drive replication. These data imply that a sequence-specific interaction with another viral protein is required to drive RNA replication and suggest that the proposed sites of contact reside in the carboxy-terminal two-thirds of 2B. Hybrid genomes in which either the amphipathic α-helix or the other hydrophobic domain was replaced failed to replicate. The potential contribution of these domains to the structure and functioning of protein 2B are discussed.

Introduction

The genus *Enterovirus* of the family of *Picornaviridae*, a large group of plus-strand RNA viruses, comprises polioviruses (PV), coxsackie A viruses, coxsackie B viruses (CBV), echoviruses, and several distinct enterovirus serotypes. These enteroviruses are closely related and share marked similarities with respect to virus structure, genomic organization and replicative cycle (Wimmer *et al.*, 1993). The main differences between these viruses are observed in the clinical syndromes they produce in humans, growth in particular cell lines, and the illness and pathology they produce in monkeys and suckling mice (Melnick, 1996). The differences in tropism are predominantly based on the ability to bind to specific receptors for cell entry, reflecting differences in the structural capsid proteins encoded by the P1 region of the genome, and the occurrence of host-specific *cis*-acting translational control elements in the genomic RNA (Agol *et al.*, 1996; Racaniello, 1990).

The enteroviruses can be divided into seven clusters, based on properties of genotype and function (Hyypiä *et al.*, 1997). CBV serotypes 1–6 and PV serotypes 1–3 fall into different clusters. The nonstructural proteins encoded by the P2 and P3 regions of the genomes of CBV and PV share 50 to 65 % amino acid identity. Despite the differences in primary sequence, there is a high degree of conservation of important structural domains and sequence motifs, suggesting that these proteins share similar functions in virus reproduction. Studies on the functional exchangeability of these proteins have provided experimental evidence for this suggestion. Chimeric PV genomes containing the coxsackie B3 virus (CBV3) counterpart of protein $3C^{pro}$, a proteinase that is responsible for the majority of the processing events that give rise to the production of the nonstructural proteins, demonstrated correct processing of the PV P2 and P3 region proteins (Dewalt *et al.*,

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1989). In addition, PV genomes carrying CBV4 protein $2A^{pro}$, a multifunctional protein that acts as a proteinase, a *trans*-activator of virus translation and as a component in viral RNA (vRNA) replication, gave rise to viable viruses (Lu *et al.*, 1995).

Enterovirus protein 2B is a small hydrophobic protein that is localized at the outer surface of the membrane vesicles on which vRNA replication takes place (Bienz *et al.*, 1994). The phenotypes of 2B mutants of both PV and CBV3 have implied a role of protein 2B, or possibly its precursor 2BC, in vRNA amplification (Johnson & Sarnow, 1991; Li & Baltimore, 1988; van Kuppeveld *et al.*, 1995, 1996*a*, *b*). The exact function of this protein is not yet unclear but seems to be related to its effects on cellular membranes. Expression of the 2B proteins of both PV and CBV3 in mammalian cells caused a modification in permeability of the plasma membrane and inhibition of vesicular protein transport (Doedens & Kirkegaard, 1995; van Kuppeveld *et al.*, 1997*a*), two phenomena that also occur during enterovirus infection (Carrasco *et al.*, 1993; Doedens & Kirkegaard, 1995). Recently, CBV3 protein 2B has also been shown to increase the permeability of the endoplasmic reticulum membrane (van Kuppeveld *et al.*, 1997*b*). The interaction of enterovirus protein 2B with membranes is most likely provided by the two conserved hydrophobic domains, of which one has the potential to form a cationic amphipathic α-helix (van Kuppeveld *et al.*, 1996*a*) and one (which will further be referred to as 'the second hydrophobic domain') shows characteristics typical of multimeric transmembrane helices (van Kuppeveld *et al.*, 1995). The relevance of these domains for the ability of CBV3 2B to increase membrane permeability and inhibit protein secretion has been confirmed by individual expression of 2B proteins carrying mutations in these regions (van Kuppeveld *et al.*, 1997*a*, *b*).

The aim of this study was to gain more insight into the structural requirements of enterovirus protein 2B for its function in virus reproduction. To this end, we have constructed chimeric CBV3 cDNAs that contained either the entire PV 2B protein or hybrid proteins in which specific segments of CBV3 protein 2B were substituted by the corresponding PV regions. The ability of PV 2B and the hybrid 2B proteins to drive vRNA replication and virus growth was assayed by transfection of cells with copy RNA transcripts of the chimeric cDNAs. Polyprotein synthesis and processing were studied by translation of RNA transcripts in a cell-free extract. The potential contribution of the distinct domains to the structure and function of protein 2B are discussed.

Methods

■ **Construction of unique endonuclease restriction sites in pCB3/T7.** For the introduction of PV sequences into plasmid pCB3/T7 (Klump *et al.*, 1990), which contains a cDNA of CBV3 (strain Nancy) behind a T7 RNA polymerase promoter, unique restriction sites were introduced, either alone or in combination, by site-directed mutagenesis. Mutagenesis was performed with a subgenomic pALTER phagemid clone containing the *Eco*RV fragment of pCB3/T7 [nucleotide (nt) 918–6177] using the Altered Sites *in vitro* mutagenesis system according to the manufacturer's instructions (Promega). The nucleotide sequence of the antisense synthetic oligonucleotides (Isogen Bioscience, The Netherlands) used for the introduction of unique restriction sites (underlined) immediately upstream or downstream of the 2B-encoding sequence are; 5′ TTCCACATAGTCCTTCAAGGCCTGTTCCATTGCATC 3′ (*Stu*I site at nt 3743) and 5′ TTTCTTAAGCCAGCTGTTAACTTGGCGTTCAGCCAT 3′ (*Hpa*I site at nt 4042). Nucleotide sequences of the oligonucleotides used for the introduction of unique restriction sites at 2B amino acids (aa) 57–59 are; 5′ TAGTGTGGCAGTCACAGTGATCAGGTCGTGGTTCCGGACCACAATTACTAAGGCTG ATAT 3′ (*Acc*III site at nt 3913) and 5′ GATCAGGTCATCGTGGTTAACCACCACAA TTACTAAGGC 3′ (*Hpa*I site at nt 3915). Mutant pALTER clones were identified by restriction enzyme analysis. The nucleotide sequence of the mutant clones was verified by dideoxy chain-termination sequencing of plasmid DNA using the Ampli Cycle sequencing kit according to the manufacturer's instructions (Perkin-Elmer). The unique restriction sites were introduced in pCB3/T7 by cloning of the *Bgl*II (nt 2040)–*Bss*HII (4238) fragments in plasmids from which the corresponding fragment was deleted. In this way, plasmids pCB3/T7-*Stu*I(3743), pCB3/T7-*Hpa*I(4042), pCB3/T7-*Hpa*I(3915), pCB3/T7-*Stu*I(3743)/*Hpa*I(4042), pCB3/T7-*Stu*I(3743)/*Hpa*I(3915) and pCB3/T7-*Acc*III(3913)/*Hpa*I(4042) were generated.

■ **Construction of chimeric cDNAs.** Chimeric cDNAs were constructed by PCR amplification and cloning of PV 2B-encoding segments into pCB3/T7 plasmids from which the corresponding segment was deleted. Plasmid pXpA (generously provided by R. Andino, University of California), which contains a cDNA of PV type 1, was used as template for PCR. A total of four forward primers (f1–f4) and five reverse primers (r1–r5) containing restriction sites at their 5′ end (underlined) were used for amplifying PV sequences. PCR was performed using Super*Taq* DNA polymerase (HT Biotechnology) according to the manufacturer's instructions. The chimeric cDNAs were named according to the CBV3 amino acids that were replaced by the corresponding residues of PV. The construction of each of the chimeric plasmids is briefly summarized:

2B/PV1-99; a DNA fragment containing the coding sequence of PV aa 1–99 was generated by PCR using primers f1 (5′ ATGGAACTGCGCATCACCAATTACATA 3′; *Avi*II site) and r1 (5′ CCAACTAATATTTTGCTTGATGACATAAGG 3′; *Ssp*I site), digested with *Avi*II and *Ssp*I, and cloned in pCB3/T7-*Stu*I(3743)/*Hpa*I(4042) from which the *Stu*I–*Hpa*I fragment was deleted.

2B/PV1-94; a DNA fragment containing the coding sequence of PV aa 1–94 was generated by PCR using primers f1 and r2 (5′ CCAACTAATATTTTGGCGTTCAGCCATAGGTATCTCCAGAACATCGCA 3′; *Ssp*I site), digested with *Avi*II and *Ssp*I, and cloned in pCB3/T7-*Stu*I(3743)/*Hpa*I(4042) from which the *Stu*I–*Hpa*I fragment was deleted.

2B/PV1-30; a DNA fragment containing the coding sequence of PV aa 1–30 was generated by PCR using primers f1 and r3 (5′ GGTACTGACTAGTGAATTGGTCAACTCTGTTAT 3′; *Spe*I site), digested with *Avi*II and *Spe*I, and cloned in pCB3/T7-*Stu*I(3743) from which the *Stu*I–*Spe*I (3837) fragment was deleted.

2B/PV1-59; a DNA fragment containing the coding sequence of PV aa 1–59 was generated by PCR using primers f1 and r4 (5′ TTCATAAGGCCTAGTTATAATAACTAG 3′; *Stu*I site), digested with *Avi*II and *Stu*I, and cloned in pCB3/T7-*Stu*I(3743)/*Hpa*I(3915) from which the *Stu*I–*Hpa*I fragment was deleted.

2B/PV60-99; a DNA fragment containing the coding sequence of PV aa 60–99 was generated by PCR using primers f2 (5′ ATTATAATCCGGAACTATGAAGACACC 3′; *Acc*III site) and r1, digested with

*Acc*III and *Ssp*I, and cloned in pCB3/T7-*Acc*III(3913)/*Hpa*I(4042) from which the *Acc*III–*Hpa*I fragment was deleted.

2B/PV60-94; a DNA fragment containing the coding sequence of PV aa 60–94 was generated by PCR using primers f2 and r2, digested with *Acc*III and *Ssp*I, and cloned in pCB3/T7-*Acc*III(3913)/*Hpa*I(4042) from which the *Acc*III–*Hpa*I fragment was deleted.

2B/PV34-59; a DNA fragment containing the coding sequence of PV aa 34–59 was generated by PCR using primers f3 (5′ AATATGCTAGCCAGTACCATCACTGAA 3′; *Nhe*I site) and r4, digested with *Nhe*I and *Stu*I, and cloned in pCB3/T7-*Hpa*I(3915) from which the *Spe*I (3837)–*Hpa*I fragment was deleted. Due to the occurrence of a *Spe*I site in the amplicon, a *Nhe*I site (compatible ends with *Spe*I) was build in the 5′ end of primer f3. As result of this, valine 33 is altered to alanine.

2B/PV34-99; a DNA fragment containing the coding sequence of PV aa 34–99 was generated by PCR using primers f3 and r1, digested with *Nhe*I and *Ssp*I, and cloned in pCB3/T7-*Hpa*I(4042) from which the *Spe*I (3837)–*Hpa*I fragment was deleted. Due to the occurrence of a *Nhe*I site rather than a *Spe*I site in primer f3 (see above), valine 33 is changed to alanine.

2B/PV34-94; a DNA fragment containing the coding sequence of PV aa 34–94 was generated by PCR using primers f3 and r2, digested with *Nhe*I and *Ssp*I, and cloned in pCB3/T7-*Hpa*I(4042) from which the *Spe*I (3837)–*Hpa*I fragment was deleted. Due to the occurrence of a *Nhe*I site rather than a *Spe*I site in primer f3 (see above), valine 33 is changed to alanine.

2B/PV37-54; a DNA fragment containing the coding sequence of PV aa 37–54 was generated by PCR using primers f4 (5′ GAGTTGTCACTAGTGGGTCAAGACACCATCACTGAAAAGCTACTTAAG-3′; *Spe*I site) and r5 (5′ TTCATAAGGCCTCACCACAATAACTAGGGAGGATATGATCTT 3′; *Stu*I site), digested with *Spe*I and *Stu*I, and cloned in pCB3/T7-*Hpa*I(3915) from which the *Spe*I (3837)–*Hpa*I fragment was deleted. Primer r5 was designed such that the *Spe*I site occurring in the PV sequence was deleted while leaving the amino acid sequence intact.

From all mutant constructs, the entire PV segment that was introduced was confirmed by sequence analysis.

■ **Transfection of cells with copy RNA transcripts.** *In vitro* transcription of *Sal*I linearized plasmids by phage T7 RNA polymerase and transfection of 75% confluent Buffalo green monkey (BGM) cell monolayers with RNA transcripts (5 μg) using the DEAE-dextran method were performed as described previously (van Kuppeveld *et al.*, 1995). After transfection, cells were fed with minimal essential medium (MEM) containing 10% foetal bovine serum (FBS) and incubated at either 33 or 36 °C. If virus growth was observed, cultures were incubated until cytopathic effect (CPE) was complete, followed by three cycles of freezing and thawing to release intracellular viruses. Viruses were aliquoted and stored at −80 °C.

■ **Analysis of RNA synthesis by dot blot hybridization.** BGM cell monolayers were transfected with wild-type and mutant RNA transcripts as described above. At various times post-transfection, the medium was discarded and the cells were washed three times with PBS. Total RNA was isolated by a single extraction procedure with guanidium thiocyanate–phenol–chloroform as described by Chomczynski & Sacchi (1987). RNAs were denatured with formamide and formaldehyde and spotted onto Hybond nylon membranes (Amersham) according to standard procedures (Ausubel, 1990). cDNA clone pCB3/T7 was labelled with [α-^{32}P]dATP by nick-translation. Membranes were prehybridized, hybridized, washed, and analysed by autoradiography according to standard procedures (Ausubel, 1990).

■ ***In vitro* translation reactions.** Copy RNA transcripts were synthesized and translated in a single reaction using T7 TNT rabbit reticulocyte lysate (Promega) supplemented with 20% (v/v) HeLa cell initiation factors (kindly provided by J. Flanegan, University of Florida). The translation reactions (20 μl) contained 0·5 μg of circular plasmid DNA and 20 μCi of Tran^{35}S-label (a mixture of [^{35}S]methionine and [^{35}S]cysteine; ICN) and were incubated for 3 h at 30 °C. Translation products were analysed on an SDS–12·5% polyacrylamide gel (Laemmli, 1970). Gels were fixed, fluorographed and exposed to Kodak XAR film at −80 °C.

■ **Sequence verification of virus.** RNA extraction, synthesis of cDNA, amplification of the 2B coding region by PCR using primers 5′ TGGTGTCATTGGCATTGTGACCATGGGGGG 3′ (nt 3648–3677) and 5′ TTGGGATGGCGCGCTCTGCTC 3′ (nt 4231–4251), purification of the PCR products, and sequence analysis with reverse primer 5′ CCATTCAATGAATTTCTG 3′ (nt 4117–4134) were all performed as described previously (van Kuppeveld *et al.*, 1996*a*).

■ **Virus titrations.** Virus titres were determined by endpoint titration as described previously (van Kuppeveld *et al.*, 1995) and expressed in 50% tissue culture infective doses ($TCID_{50}$) according to the method of Reed & Muench (1938).

■ **Single-cycle growth analysis.** Confluent BGM cell monolayers grown in 25 cm^2 flasks (5×10^6 cells) were infected with virus at an m.o.i. of 1 $TCID_{50}$ per cell for 30 min at room temperature. The cells were fed with MEM containing 3% FBS and grown at 33, 36 or 39 °C. At various times post-infection, cells were disrupted by three cycles of freezing and thawing, and the virus titres were determined.

■ **Analysis of viral protein synthesis *in vivo*.** Confluent monolayers of BGM cells were infected with virus at an m.o.i. of 25 for 30 min at room temperature. After infection, cells were fed with MEM containing 3% FBS and grown at 36 °C. At various times post-infection, the cells were washed with PBS and incubated in methionine- and serum-free MEM (Gibco) containing 10 μCi of Tran^{35}S-label for 30 min. Lysis of cells and analysis of the [^{35}S]methionine-labelled proteins by SDS–PAGE were performed as previously described (van Kuppeveld *et al.*, 1995).

Results

Construction of heterologous CBV3 cDNAs

To allow the construction of chimeric CBV3 genomes, unique restriction sites were introduced, either alone or in combination, at the 2A/2B and 2B/2C junctions and within the 2B coding sequence of plasmid pCB3/T7. PV sequences were amplified by PCR and cloned in these constructs. PCR primers were designed such that no additional amino acid changes outside the substituted fragment were introduced. A total of ten chimeric constructs, schematically diagrammed in Fig. 1, was generated. In construct 2B/PV1-99, the complete 2B coding region of CBV3 was replaced by that of PV. In the other constructs, the amino-terminal region (i.e. the first 30–35 amino acids), the amphipathic α-helix, or the second hydrophobic domain were replaced, either alone or in combination, by their corresponding PV counterparts. Because the borders of the amphipathic α-helix are not known exactly, two different constructs (2B/PV34-59 and 2B/PV37-54) were generated. Constructs 2B/PV1-99, 2B/PV60-99 and 2B/PV34-99 contained at the P4 position of the 2B/2C cleavage site a valine

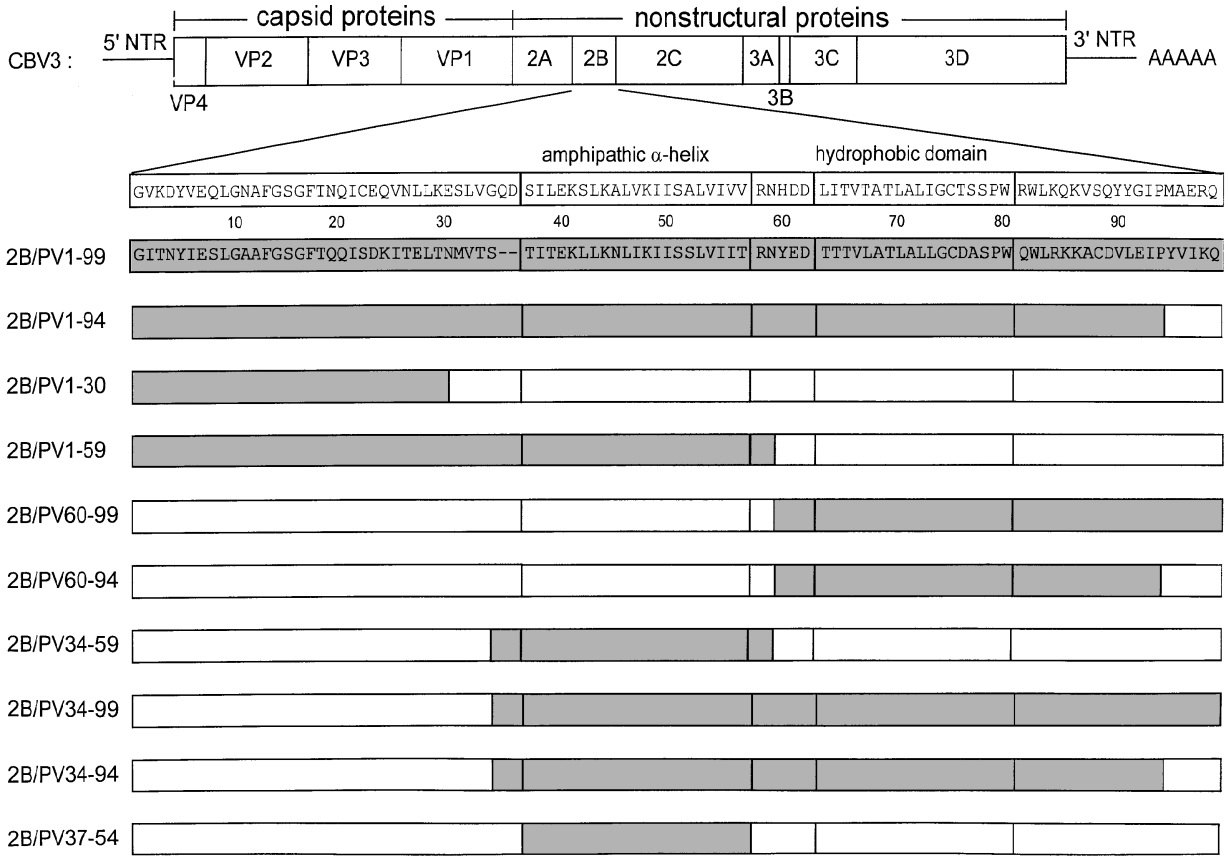

Fig. 1. Schematic representation of the chimeric CBV3 genomes. At the top of the figure is shown the 7·5 kb single-stranded RNA genome of CBV3 with the polyadenylate tract at the end of the 3′ nontranslated region (NTR). The boxed region shows the polyprotein and vertical lines within the box indicate proteinase cleavage sites. The complete amino acid sequence of protein 2B, containing the cationic amphipathic α-helix and the second hydrophobic domain, is enlarged. The poliovirus 2B encoding segments are shaded.

rather than an alanine (which occurs in wild-type CBV3). Therefore, three additional constructs (2B/PV1-94, 2B/PV60-94 and 2B/PV34-94) containing the last five amino acids of CBV3 2B were generated as well.

Viability of chimeric CBV3 genomes

To examine whether PV protein 2B and the hybrid PV–CBV3 2B proteins could functionally replace the 2B protein of CBV3 *in vivo*, BGM cells were transfected with *in vitro* synthesized RNA transcripts from wild-type pCB3/T7 and the chimeric constructs. Four transfections were performed for each construct. Two transfected cell cultures were grown at 33 °C and two were grown at 36 °C. If no virus growth was observed after 5 days, the cultures were subjected to three cycles of freezing and thawing, and passaged to fresh cell cultures, which were incubated for an additional 3 days.

Transfection of cells with RNA transcripts derived from the wild-type plasmid gave complete CPE within 2 days. RNA transcripts from construct 2B/PV1-30 produced CPE in all transfected cell cultures at 5 days post-transfection. Sequence analysis of the 2B coding region of this virus (vCB3-2B/PV1-30) showed that the introduced PV segment was retained and that no other mutations had occurred. No CPE was observed in any of the cell cultures that were transfected with RNA of the other chimeras. Passage of cytoplasmic extracts to fresh BGM cell monolayers also failed to reveal virus growth.

Analysis of the virus growth of vCB3-2B/PV1-30 in a single-cycle infection showed a delay in virus production relative to wild-type virus (Fig. 2). This delay was most apparent at 6 h post-infection, when mutant virus production was only 1% of that of wild-type virus. The delay in virus growth was not temperature sensitive; the kinetics of virus reproduction of vCB3-2B/PV1-30 relative to that of wild-type virus was similar at 33, 36 and 39 °C (data not shown).

Analysis of vRNA replication

To examine whether the reduced growth of vCB3-2B/PV1-30 and the nonviability of the other chimeric genomes were

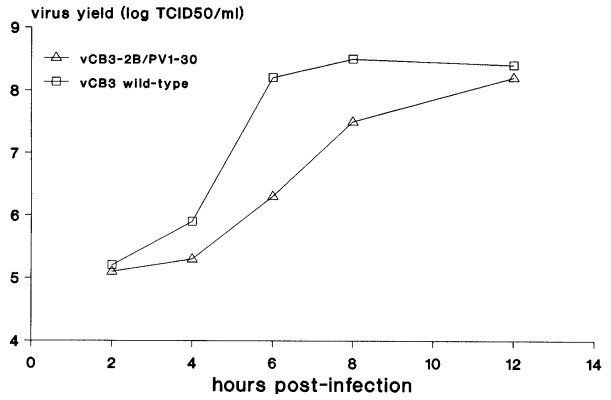

Fig. 2. One-step growth curves of wild-type virus and mutant virus vCB3-2B/PV1-30. BGM cell monolayers were infected at a multiplicity of 1 $TCID_{50}$ per cell and incubated at 36 °C. At the indicated times post-infection, the cells were disrupted by three cycles of freezing and thawing and virus production was determined by endpoint titration.

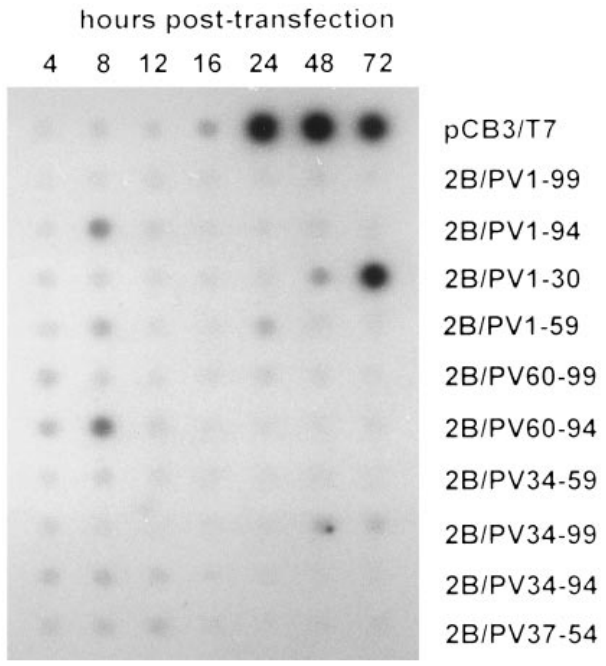

Fig. 3. Analysis of wild-type and mutant viral RNA replication. BGM cell monolayers were transfected with equal amounts of genomic transcripts derived from the indicated plasmids linearized by *Sal*I. At the indicated times post-transfection, total RNA was isolated, denatured, bound to nylon membrane and hybridized to an α-^{32}P-labelled cDNA probe.

due to a defect in vRNA replication, a dot blot hybridization was performed. BGM cells were transfected with *in vitro* synthesized copy RNA transcripts and at various times post-transfection total cellular RNA was isolated, denatured, immobilized on nylon membranes and hybridized with a [α-^{32}P]dATP-labelled CBV3 cDNA probe. Fig. 3 shows that there was no detectable vRNA synthesis in cells transfected with any of the nonviable chimeras. From the hybridization signals obtained with construct 2B/PV1-30 it is evident that vRNA synthesis is delayed.

Synthesis and processing of hybrid polyproteins

To examine whether the chimeric genomes were able to correctly generate all nonstructural and structural proteins required to replicate and assemble viral RNAs, copy RNAs of the mutant constructs were synthesized and translated in a reticulocyte lysate. Fig. 4 shows that all mutant RNAs produced cleavage products to levels similar to those produced by RNA derived from pCB3/T7. Protein 2B (11 kDa) could not be visualized because it migrated in the heavily overloaded globin spot. Nevertheless, the efficient production of proteins 2A and 2C indicated that none of the introduced PV segments interfered with cleavage site specificity and processing efficiency of $3C^{pro}$ at either the 2A/2B or 2B/2C junction. Thus, the defects in vRNA replication and virus growth of the chimeric genomes are unlikely to be due to impaired processing of the viral polyprotein.

To examine the possibility that the defect in vRNA replication of vCB3-2B/PV1-30 was due to a reduction in the rate of viral protein synthesis *in vivo*, we compared protein synthesis following infection of BGM cells with either wild-type or the chimeric virus. Cells were infected at an equal m.o.i. and pulse-labelled with [^{35}S]methionine at various times post-infection. Analysis of the cell lysates shows that the chimeric virus has retained the ability to shut off cellular protein synthesis (Fig. 5). In chimeric virus-infected cells, viral protein synthesis was maximal at 7 h post-infection. The amount of viral proteins produced at this time-point was similar to that observed in wild-type virus-infected cells at 5 h post-infection, when wild-type protein synthesis was maximal. This suggests that it is unlikely that the defect in vRNA replication is due to an impaired rate of viral protein synthesis *in vivo*.

Discussion

The construction of chimeric viral genomes is a suitable approach to dissect multiple functions of specific genetic elements. Hybrid picornavirus genomes have provided interesting information on the occurrence of independent functional domains essential for vRNA replication and translation in both the 5′ nontranslated region (Alexander *et al.*, 1994; Rohll *et al.*, 1994; Xiang *et al.*, 1995) and nonstructural protein $2A^{pro}$ (Lu *et al.*, 1995). We have employed a chimeric cDNA approach to dissect the structural requirements of enterovirus protein 2B for its functioning in vRNA replication. Chimeric CBV3 genomes were constructed that expressed either the entire PV 2B protein, which is 50% homologous to the 2B protein of CBV3, or hybrid proteins in which specific structural elements of CBV3 2B were substituted by their PV counterpart. All hybrid polyproteins were efficiently synthesized and correctly processed. Defects in vRNA replication are therefore primarily attributable to impaired functioning of the 2B protein.

A chimeric CBV3 genome that expressed the entire PV 2B

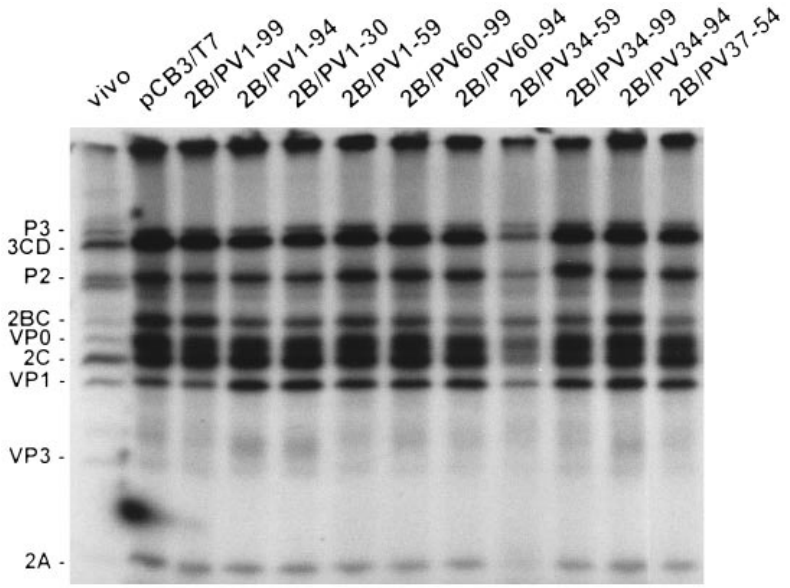

Fig. 4. *In vitro* translation of wild-type and mutant RNAs in a cell-free extract. RNA transcripts were synthesized and translated in TNT rabbit reticulocyte lysate, a coupled transcription/translation system, supplemented with HeLa cell initiation factors. Reactions were programmed with 0·5 μg of circular plasmid DNA and incubated for 3 h at 30 °C. The [^{35}S]methionine-labelled translation products were analysed on an SDS–12·5% polyacrylamide gel. An extract from wild-type virus-infected cells, labelled with [^{35}S]methionine at 4 h post-infection, was used as marker (lane 'vivo').

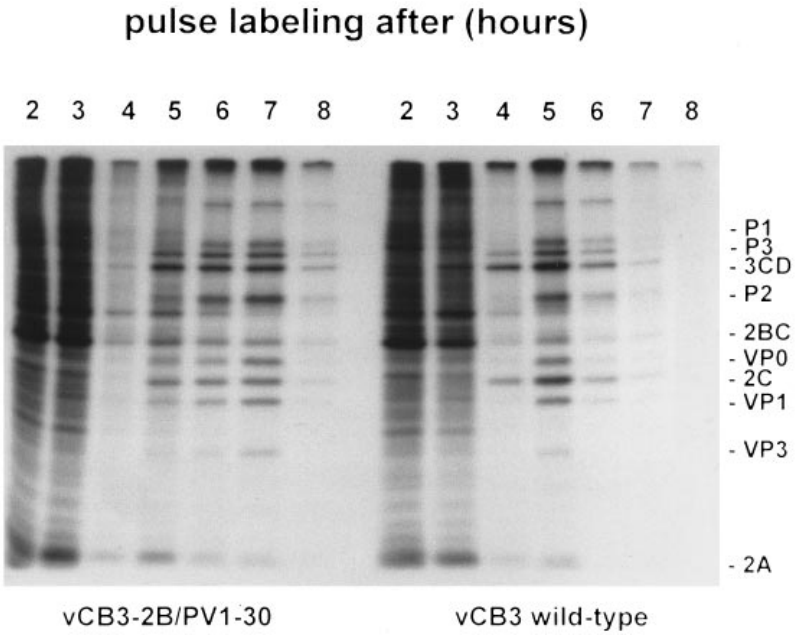

Fig. 5. Protein synthesis in wild-type virus- and vCB3-2B/PV1-30-infected cells. BGM cells were infected at a multiplicity of 25 $TCID_{50}$ per cell and grown at 36 °C. At the indicated times post-infection, cells were radioactively labelled in 30 min pulses with [^{35}S]methionine. Cellular extracts were prepared and analysed on an SDS–12·5% polyacrylamide gel.

protein failed to replicate. That PV 2B cannot functionally replace CBV3 2B is remarkable because these proteins are endowed with the same biochemical activities. Expression in mammalian cells of the 2B proteins of both PV (Doedens & Kirkegaard, 1995) and CBV3 (van Kuppeveld *et al.*, 1997*a*, *b*) results in modification of plasma membrane permeability and inhibition of protein secretion. Modification of membrane permeability is also observed when these 2B proteins are expressed in *E. coli* cells (Lama & Carrasco, 1992; F. J. M. van Kuppeveld & W. J. G. Melchers, unpublished results). The

exchangeability of the $2A^{pro}$ and $3C^{pro}$ proteins of PV with those of CBVs (Dewalt *et al.*, 1989; Lu *et al.*, 1995) and the overall similarity in structure of the nonstructural enterovirus proteins are further indicative of a conservation of the functions of these proteins. Alternative explanations for the non-exchangeability of protein 2B must therefore be considered. A possible cause for the defect in vRNA replication of this chimera may be the heterologous nature of protein 2BC, a relatively stable processing intermediate that is required for the induction of the membrane vesicles on which vRNA amplification takes place (Barco & Carrasco, 1995; Bienz *et al.*, 1983). Impaired functioning of a heterologous precursor protein was also observed with PV chimeras that contained $3C^{pro}$ of either human rhinovirus 14 or CBV3. These 3C proteinases were able to cleave the nonstructural precursor polypeptides, but the hybrid $3CD^{pro}$ proteins were unable to process the PV capsid proteins (Dewalt *et al.*, 1989). Another possible explanation for the inability of PV 2B to drive vRNA synthesis may be a failure to recognize and contact other CBV3 replication proteins. Thus, apart from its ability to modify membrane permeability and inhibit protein secretion, protein 2B may play a direct role in vRNA replication in which interactions with other viral proteins are essential. This suggestion is consistent with the existence of mutations that interfere with vRNA replication but that do not impair the ability of 2B to increase membrane permeability or inhibit protein secretion (van Kuppeveld *et al.*, 1997*a*).

Only one of the hybrid 2B proteins was functional in vRNA replication. This hybrid protein (2B/PV1-30) contained the amino-terminal one-third of PV 2B, i.e. the region upstream of the hydrophobic domains, and the remainder of CBV3 2B. Remarkably, a hybrid protein that contained the amino-terminal one-third of CBV3 2B and the remainder of PV 2B (2B/PV34-99) was non-functional. The loss of activity of this protein may be due to a general disruption of the structure of either 2B or 2BC. However, this possibility seems unlikely in view of the correct functioning of protein 2B/PV1-30. The explanation that the carboxy-terminal two-thirds of the protein contains the sequence-specific determinants required for an intramolecular interaction with 2C (in precursor 2BC) or for intermolecular contacts with other viral replication proteins seems more plausible and is in agreement with the non-functioning of PV 2B in vRNA replication, as discussed above.

Hybrid genomes that contained the cationic amphipathic α-helix of PV 2B (2B/PV34-59, 2B/PV37-54, 2B/PV1-59) failed to replicate. This is in agreement with the previously described defect in vRNA replication of 2B-bomII, a chimeric CBV3 genome that produced a 2B protein in which the cationic amphipathic helix was replaced with a similar motif of the lytic peptide bombolitin II (van Kuppeveld *et al.*, 1996*a*). The amphipathic helix motif in CBV3 2B is a major determinant for the ability to modify membrane permeability and inhibit protein secretion (van Kuppeveld *et al.*, 1997*a*, *b*). The occurrence of such a motif is characteristic for membrane-disrupting proteins. Two models of action have been proposed to explain the membrane-perturbing activities of these proteins (Bernheimer & Rudy, 1986; Shai, 1995). According to the first model, the amphipathic helix lies collateral to the membrane and inserts a few Ångstroms into it, thereby making the membrane phospholipids more susceptible to degradation by phospholipases. The second model suggests that the amphipathic helices form aqueous pores by spanning the membrane and forming oligomers, exposing their hydrophobic faces to the lipid bilayer and their hydrophilic faces forming the aqueous interior. The non-exchangeability of the amphipathic helix argues against an independent role (i.e. the first model) of this domain in the structure and function of 2B. The reason for the non-functioning of these hybrid 2B proteins is unknown. A possible explanation is that the amphipathic helix and the second hydrophobic domain of CBV3 2B are cooperatively involved in the formation of membrane-integral pores. Disturbances of contacts between these two domains may also account for the nonviability of the constructs that contained the amphipathic helix of CBV3 2B but the second hydrophobic domain of PV 2B (2B/PV60-99, 2B/PV60-94). Alternatively, the non-functioning of these hybrid proteins may be due to general disruption of 2B structures.

In conclusion, these studies have allowed us to separate the contribution of distinct domains to the function of protein 2B in vRNA replication. We have shown that the amino-terminal one-third of CBV3 2B, but not the middle one-third or the carboxy-terminal one-third of the protein, neither alone nor in combination, can be functionally replaced with its PV counterpart. In contrast to the carboxy-terminal two-thirds of the protein, the role of the amino-terminal region in the function of 2B protein is yet unclear. Mutations in this region caused defects in vRNA replication (Johnson & Sarnow, 1991), but did not interfere with the ability of protein 2B to inhibit protein secretion (van Kuppeveld *et al.*, 1997*a*). Recently, it has been shown that deletion of the first 30 amino acids of protein 2B abolished the ability of PV protein 2BC to induce membrane proliferation and to interfere with the exocytic pathway in yeast cells (Barco & Carrasco, 1995). The importance of this region for a function of protein 2B, or 2BC, in mammalian cells awaits further investigation.

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